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2. Patent application number
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3. Full name, address and postcode of the or of each applicant *(underline all surnames)* — BioXell S.p.A.
Via Olgettina 58
I-20132 Milan
Italy

- 3 NOV 2003

Patents ADP number *(if you know it)* — 8584972001

If the applicant is a corporate body, give the country/state of its incorporation — Italy

4. Title of the invention — COMPOUND AND USE IN TREATMENT

5. Name of your agent *(if you have one)* — BOULT WADE TENNANT

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)* — VERULAM GARDENS
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51/77

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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11. I/We request the grant of a patent on the basis of this application.

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COMPOUND AND USE IN TREATMENT

The present invention is concerned with the use of 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol (Compound A) for the manufacture of a medicament for the prevention and/or treatment of benign prostatic hyperplasia (BPH) and associated symptoms. It is further concerned with a method for preventing and/or treating benign prostatic hyperplasia and associated symptoms by administering 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol in an amount effective to prevent and/or to treat such disease alone or in combination with further active agents.

Benign Prostatic Hyperplasia is a common disorder in elderly men, occurring in approximately 50% of men aged 60 years and in 90% of those aged 85 years. BPH is a specific histopathological entity characterized by stromal and epithelial cell hyperplasia.

For over a century, the two known etiologic factors for the pathogenesis of BPH have been aging and the presence of functional testes. However, as the science of prostate biology advances, this concept becomes inadequate as it does not cover all aspects of BPH pathogenesis. Additional etiologic factors play a significant role in regulating prostatic growth. In particular, evidence has emerged that prostatic growth is under the immediate control of specific growth factors produced by prostatic cells, acting locally on adjacent cells in a paracrine mechanism or to the same cells in an autocrine mechanism. Therefore much effort is currently being put into identifying therapeutic strategies aimed at inhibiting intraprostatic growth factors.

BPH is a common cause of chronic lower urinary tract symptoms which may affect both the filling (irritative symptoms) and voiding (obstructive symptoms) phases of the micturition cycle. These symptoms affect the social,

psychological, domestic, occupational, physical and sexual lives of the patients leading to a profound, negative impact on their quality of life. In addition to this, BPH can cause more acute urological complications, particularly acute urinary retention (AUR), often considered the most serious complication of BPH and less frequently recurrent urinary tract infections, upper urinary tract dilatation, bladder stone formation and recurrent hematuria.

BPH management is associated with extremely high social costs, estimated to be 4 billion dollars in 1993 and projected to be 26 billion dollars in 2003 in the USA alone.

The current medical treatment for BPH has consists of orally administered 5 alpha reductase inhibitors (Finasteride and Dutasteride, recently approved by the FDA) and alpha 1 receptor antagonists (Terazosin, Doxazosin, Tamsulosin etc.). Each of these therapeutic options is associated with both advantages and a disadvantages relating to their different mechanism of action. While alpha 1 receptor antagonists are very effective in reducing symptoms related to lower urinary tract symptoms (LUTS), they are ineffective in reducing the prostate volume and therefore in preventing BPH-related surgery. Conversely, 5 alpha reductase inhibitors like finasteride and dutasteride, by decreasing dihydrotestosterone (DHT) formation, reduce prostate size and the need for surgery. In addition, recent results from the seven-year Prostate Cancer Prevention Trial, involving more than 18.000 healthy aged man, demonstrated that finasteride can prevent or delay the appearance of prostate cancer (see Thompson IM, *et al. New England Journal of Medicine* (2003) 349 p 215-224). However, as expected (see Kassabian VS. *Lancet* (2003) 361 p 60-62), finasteride was not free from anti-androgenic adverse effects on sexual function, such as decreased sexual potency, sexual desire and gynecomastia, that substantially lessen its

probably because the finasteride-induced low androgen state selected the most aggressive, androgen-insensitive malignant growing cells (see Scardino PT *New England Journal of Medicine* (2003) *349* p 297-299.).

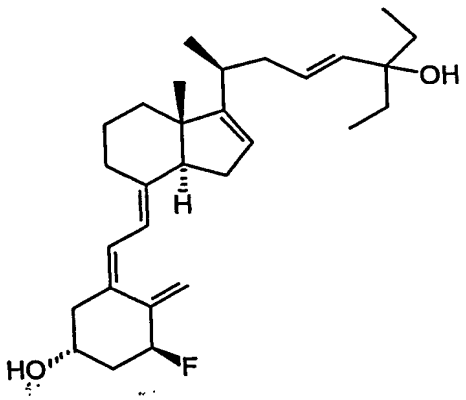
Thus there is an unmet need for a new class of drugs for medical therapy of BPH, which should be able to prevent acute urinary retention, together with its related need for surgery, by decreasing androgen-induced prostate growth but without directly interfering with androgen receptors (AR), and therefore without anti-androgenic prostatic and extra-prostatic adverse effects, for example, sexual side effects. Such medicaments, by disrupting intra-prostatic growth factor signalling, might be useful not only for treating BPH but also for preventing prostate cancer, possibly without selecting AR-insensitive, malignant clones.

As described herein, the Inventors have determined that the non-hypercalcemic, well-tolerated, vitamin D_3 analogue 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol (compound A), is a primary example of such a medicament, as it can act against BPH in an androgen-receptor independent manner by targeting growth factor-mediated prostate proliferation.

1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], the activated form of vitamin D_3, is a secosteroid hormone that not only plays a central role in bone and calcium metabolism, but is also involved in the regulation of the immune response and the differentiation and apoptosis of many cell types, including malignant cells.

However, a problem with the therapeutic use of calcitriol is its natural ability to induce hypercalcemia and hyperphosphatemia. Hence, analogues of calcitriol retaining biological activity but devoid of hypercalcemic side effects, have been developed.

It has now surprisingly been found in several studies, conducted by the Inventors, that the $1,25(OH)_2D_3$ analogue Compound A:



Compound A

significantly reduces the growth of human BPH cells *in vitro* via induction of their apoptosis and reduces prostatic growth *in vivo*, with no effects on testosterone and dihydrotestosterone levels. Furthermore, this inhibition of prostate growth is achieved at non-hypercalcemic doses. Thus, Compound A is an effective pharmacologic agent for the treatment of benign prostatic hyperplasia.

As described in the Examples herein, Compound A reduces prostate size to a extent similar to finasteride. Furthermore, as observed with finasteride, Compound A counteracts against the *in vitro* and *in vivo* proliferative activity of testosterone. Significantly however, and unlike finasteride, Compound A does not inhibit type-1 or type-2 5 α-reductase activity and can counteract not only testosterone but even dihydrotestosterone induced BPH cell growth. These anti-androgenic properties of Compound A are independent from interaction with the AR, as shown by the failure of Compound A both to bind to the AR, or to act as an AR agonist or antagonist. Furthermore, Compound A does not affect gonadal hormone secretion.

Thus, in a first aspect, the invention provides the use of 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol for the manufacture of medicaments for the prevention and/or treatment of benign prostatic hyperplasia. Also considered within the scope of the invention are pharmaceutically acceptable esters and salts of compound A.

1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol is a known compound and its preparation is described in US Patent 5,939,408, the description of which is incorporated herein by reference.

In a second aspect, the present invention provides a method for preventing and/or treating benign prostatic hyperplasia, in patients in need of such treatment comprising administering per os, parenteral (including subcutaneous, intramuscular and intravenous), rectal, buccal (including sublingual), pulmonary, transdermal, and intranasal, most preferably oral, an amount of 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol effective to prevent and/or treat the disease.

In a third aspect, the present invention provides a packaged formulation which includes a pharmaceutical composition comprising 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol and a pharmaceutically-acceptable carrier packaged with instructions for use in the treatment of benign prostatic hyperplasia.

The compound can be used as a monotherapy or it can be administered in combination with known BPH-active agents for example an alpha-adrenergic receptor blocking agent such as an alpha 1 receptor antagonist (for example Terazosin, Doxazosin or Tamsulosin). The combination partner can be admixed with the compound or its salts in various ratios or can be administered separately or sequentially.

Dosage levels and time course of administration of the active ingredients in the pharmaceutical compositions of the invention may be varied

so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient. An exemplary dose range is from 0.1 to 300 µg per day, for example 50-150 µg per day.

Specifically, a preferred dose of Compound A is the maximum that a patient can tolerate and not develop hypercalcemia. Preferably, the vitamin D compound of the present invention is administered at a concentration of about 0.001 µg to about 100 µg per kilogram of body weight, about 0.001 – about 10 µg/kg or about 0.001 µg – about 100 µg/kg of body weight. Ranges intermediate to the above-recited values are also intended to be part of the invention.

This dosage may be delivered in a conventional pharmaceutical composition by a single administration, by multiple applications, or via controlled release, as needed to achieve the most effective results, preferably once or twice daily by mouth. In certain situations, alternate day dosing may prove adequate to achieve the desired therapeutic response.

The selection of the exact dose and composition and the most appropriate delivery regimen will be influenced by, *inter alia*, the pharmacological properties of the formulation, the nature and severity of the condition being treated, and the physical condition and mental acuity of the recipient.

Representative delivery regimens include oral, parenteral (including subcutaneous, intramuscular and intravenous), rectal, buccal (including sublingual), pulmonary, transdermal, and intranasal, most preferably oral. Administration may be continuous or intermittent (e.g., by bolus injection).

A further aspect of the present invention relates to pharmaceutical compositions comprising as an active ingredient a compound of the present

(subcutaneous, intramuscular or intravenous) administration, particularly in the form of liquid solutions or suspensions; for oral or buccal administration, particularly in the form of tablets or capsules; for pulmonary or intranasal administration, particularly in the form of powders, nasal drops or aerosols; and for rectal or transdermal administration.

The compositions may conveniently be administered in unit dosage form and may be prepared by any of the methods well-known in the pharmaceutical art, for example as described in Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Company, Easton, PA., (1985). Formulations for parenteral administration may contain as excipients sterile water or saline, alkylene glycols such as propylene glycol, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, hydrogenated naphthalenes and the like. Formulations for nasal administration may be solid and may contain excipients, for example, lactose or dextran, or may be aqueous or oily solutions for use in the form of nasal drops or metered spray. For buccal administration typical excipients include sugars, calcium stearate, magnesium stearate, pregelatinated starch, and the like.

Orally administrable compositions may comprise one or more physiologically compatible carriers and/or excipients and may be in solid or liquid form. Tablets and capsules may be prepared with binding agents, for example, syrup, acacia, gelatin, sorbitol, tragacanth, or poly-vinylpyrollidone; fillers, such as lactose, sucrose, corn starch, calcium phosphate, sorbitol, or glycine; lubricants, such as magnesium stearate, talc, polyethylene glycol, or silica; and surfactants, such as sodium lauryl sulfate. Liquid compositions may contain conventional additives such as suspending agents, for example sorbitol syrup, methyl cellulose, sugar syrup, gelatin, carboxymethylcellulose, or edible fats; emulsifying agents such as lecithin, or acacia; vegetable oils such as almond oil, coconut oil, cod liver oil, or peanut oil; preservatives such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT).

Liquid compositions may be encapsulated in, for example, gelatin to provide a unit dosage form.

Preferred solid oral dosage forms include tablets, two-piece hard shell capsules and soft elastic gelatin (SEG) capsules. SEG capsules are of particular interest because they provide distinct advantages over the other two forms (see Seager, H., "Soft gelatin capsules: a solution to many tableting problems"; Pharmaceutical Technology, 9, (1985). Some of the advantages of using SEG capsules are: a) dose-content uniformity is optimized in SEG capsules because the drug is dissolved or dispersed in a liquid that can be dosed into the capsules accurately b) drugs formulated as SEG capsules show good bioavailability because the drug is dissolved, solubilized or dispersed in an aqueous-miscible or oily liquid and therefore when released in the body the solutions dissolve or are emulsified to produce drug dispersions of high surface area and c) degradation of drugs that are sensitive to oxidation during long-term storage is prevented because the dry shell of soft gelatin provides a barrier against the diffusion of oxygen.

The dry shell formulation typically comprises of about 40% to 60% concentration of gelatin, about a 20% to 30% concentration of plasticizer (such as glycerin, sorbitol or propylene glycol) and about a 30 to 40% concentration of water. Other materials such as preservatives, dyes, opacifiers and flavours also may be present. The liquid fill material comprises a solid drug that has been dissolved, solubilized or dispersed (with suspending agents such as beeswax, hydrogenated castor oil or polyethylene glycol 4000) or a liquid drug in vehicles or combinations of vehicles such as mineral oil, vegetable oils, triglycerides, glycols, polyols and surface-active agents.

In an example formulation, the soft gelatin capsules are size 2, white, opaque, oval gelatin capsules containing a liquid fill consisting of the active

butylated hydroxyanisole (BHA), NF, as preservatives. The soft gelatin capsules can be formulated to contain 150 µg of Compound A. Soft gelatin capsules should be stored at 2 – 8°C and protected from light.

The present invention will now be described with reference to the following non-limiting examples, with reference to the figures, in which:

Figure 1. shows inhibition of BPH cell proliferation by calcitriol and Compound A ("Cmpd A"). *Panel A* Incubation for 48 h with increasing concentrations (10^{-18}-10^{-7} M) of calcitriol (circles) or Compound A (squares) resulted in a significant and dose-dependent inhibition of BPH cell growth (*P<0.01 vs control). ALLFIT analysis indicates that the two secosteroids share the same maximal inhibition (I_{max}=43±1%), but show a marked difference in the rank of potency (-logIC_{50} Compound A=15.8±0.3; -logIC_{50} calcitriol=10.2±0.6, P<0.005). Results are expressed as % inhibition (mean±SEM) over their relative controls in 3 different experiments performed in triplicate. *Panel B* Effect of increasing concentrations (10^{-18}-10^{-7} M) of Compound A on BPH cell proliferation stimulated by T (10 nM, squares), KGF (10 ng/ml, circles) or Des(1-3)IGF-I (10 ng/ml, triangles). Compound A induced a significant inhibition (*P<0.01 vs T- or GF-treated cells) of BPH cell growth also in presence of all the stimuli tested with similar I_{max}=66.6±7.3%. However, Compound A was more potent in inhibiting the effect of T (-log IC_{50}=16.4±0.6), than of the other two GFs, (-log IC_{50} Des(1-3)IGF-I =12.7±0.6, and -log IC_{50} KGF=14.2±0.6, P<0.0001). Results are expressed as % variation (mean±SEM) over the maximal stimulation in 3 different experiments performed in triplicate.

Figure 2. shows the effect of Compound A, cyproterone acetate and finasteride on androgen-stimulated BPH cell growth. BPH cells were incubated for 48 h with Compound A (1 nM) or anti-androgens (finasteride, F, 1 nM; cyproterone acetate, Cyp, 100 nM) in the presence of T (10 nM, *Panel A*) or

DHT (10 nM, *Panel B*). Results obtained in unstimulated BPH cells are also shown (*Panel A*). Results are expressed as % variation (mean±SEM) over their relative controls in three different experiments performed in quadruplicate. Compound A and cyproterone acetate significantly blocked both T- and DHT-induced growth, while finasteride was effective only against T. *P<0.01 vs control; °P<0.01 vs androgen-treated cells.

Figure 3. shows the lack of agonistic or antagonistic properties of Compound A on human AR.The AR deficient PC3 cell line stably transfected with the human AR was plated in 24 well plates at a density of $2x10^4$ cells/well. After 24 h, the cells were transfected with the AR-responsive plasmid pLSPP and, 48 h later, cells were incubated with increasing concentrations of DHT (squares) or Compound A (circles) (panel A), or with a fixed concentration of DHT (3 nM) in the presence of bicalutamide (squares) or Compound A (circles)(panel B) for 18 h. Results (the mean of three transfection experiments) are expressed as percentage of bioluminescence per µg of total proteins. To evaluate agonistic activity 100% luciferase activity was set in the presence of DHT 100 nM (*panel A*), whereas to test antagonistic activity 100% luciferase activity was set with DHT 3 nM (*panel B*).

Figure 4. shows inhibition of rat ventral prostate growth by Compound A or finasteride. Panel A: Castrated rats, injected with T enanthate (30 mg/Kg/week), were orally treated for 5 day/week for two consecutive weeks with vehicle or with increasing doses of Compound A (10, 30, 100 and 300 µg/Kg) or finasteride (F, 10 and 40 mg/Kg). Ventral prostate weight is expressed as % variation (mean±SEM) of the weight of intact, vehicle-treated, rats (^P<0.05, *P<0.01 vs control rats, °P<0.01 vs T-supplemented rats). Panel B: Intact adult rats were orally treated for over one month (5 times/week for a total of 27 administrations) with vehicle (control) or increasing concentrations of

Ventral prostate weight is expressed as % variation (mean±SEM) of the weight of control, vehicle-treated rats (*P<0.01 vs control rats).

Figure 5. shows the effect of Compound A and finasteride on clusterin gene expression in the rat ventral prostate. Panel A. Northern analysis of clusterin mRNA expression in the ventral prostate of vehicle-treated intact (lane 1) or orchidectomized (lane 2) rats. Lanes 3-6 show clusterin gene expression in orchidectomized rats supplemented for two weeks with T enanthate (30 mg/Kg) and orally treated with vehicle (lane 3), Compound A (300 µg/Kg, lane 4 and 100 µg/Kg, lane 5) or finasteride (40 mg/Kg, lane 6). Every lane was loaded with 10 µg of total RNA. The corresponding GAPDH expression and the ethidium bromide staining of the gel are shown below the blot. The blot is representative of two separate experiments. Panel B. Northern analysis of clusterin mRNA expression in the ventral prostate of adult intact rats orally treated for over 1 month (5 times/week, 27 administrations) with vehicle (lane 1), increasing concentrations of Compound A (10, 30, µg/Kg, lane 2 and 3) or finasteride (40 mg/Kg, lane 4). Every lane was loaded with 10 µg of total RNA. The corresponding GAPDH expression and the ethidium bromide staining of the gel are shown below the blot. The blot is representative of two separate experiments.

Figure 6. shows the morphological effects of Compound A on ventral prostate of castrated and T-supplemented rats. Panels A, B, C and E. Representative fields obtained from cross-sections of whole prostate glands immunostained with a monoclonal antibody against rat clusterin and counterstained with haematoxylin. In vehicle-treated rats castrated 4-days earlier, clusterin labelling is detectable in the cytoplasm of the atrophic cuboidal epithelial cells (Panel A, 10x). After two-week T supplementation (Panel B, 10x), almost all the clusterin labelling disappeared. Conversely, clusterin positive cells were still present in rats treated with T and different doses of Compound A (100 µg/Kg, Panel C; 300 µg/Kg, Panel E, black

arrows). Panels D and F shows sections consecutive to those in Panel C and E, to highlight DNA fragmentation as assessed by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labelling (TUNEL). Two-week treatment (9 administrations) with Compound A (100 µg/Kg, Panel D; 300 µg/Kg, Panel F) induced massive apoptosis in the majority of epithelial and stromal cells. Note (black arrows), that all the clusterin-positive cells were undergoing apoptosis, while also a consistent portion of clusterin unlabeled cells shows nuclear fragmentation.

Figure 7. shows the morphological effects of Compound A and finasteride on the prostate of intact, adult rats. Panels A, B, D, E, F and H. Representative fields obtained from cross-sections of whole prostate glands immunostained with a monoclonal antibody against rat clusterin and counterstained with haematoxylin. In Panel A (10x) the primary antibody was omitted.. Panel B (10x) shows that in untreated adult rats only few, scanty epithelial cells were labelled in some glands (black arrows). Conversely, in prostate glands from rats treated with increasing concentrations of Compound A cuboidal epithelial cells showing the hallmark of atrophy were dose-dependently stained for clusterin (see black arrows, 10 µg/Kg, Panel D; 30 µg/Kg, Panel E, 100 µg/Kg, Panel F, 10x). Similar results were obtained with finasteride (40 mg/Kg, Panel H, 10x). Panels C, G and I shows serial, consecutive slices to those depicted in Panel B, D and F, respectively, to highlight DNA fragmentation as assessed by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labelling (TUNEL). Note (black arrows), that all the clusterin-positive cells were undergoing apoptosis, while also a consistent portion of clusterin unlabeled cells shows nuclear fragmentation.

Figure 8. shows results of a chronic toxicity study in dogs. A clear reduction of prostate weight is shown after 9 months of treatment with [illegible]

Figure 9. shows results of a chronic toxicity study in dogs. A reduction of prostate weight after recovery from treatment with Compound A relative to placebo.

EXAMPLES

Example 1: Effects of Compound A on BPH cells in vitro

MATERIALS AND METHODS

Materials

Minimum Essential Medium (MEM), DMEM-F12 1:1 mixture, Ham's F12 medium, phosphate buffered saline (PBS), bovine serum albumin (BSA) fraction V, glutamine, geneticine, collagenase type IV, vitamin D_3, testosterone (T), dihydrotestosterone (DHT), cyproterone acetate, β-nicotinamide adenine dinucleotide 3'-phosphate reduced form (NADPH), dithithreitol (DTT), phenylmethylsulfonyl fluoride (PMSF) and a kit for measuring calcemia were purchased from Sigma (St. Louis, MO). The protein measurement kit was from Bio-Rad Laboratories, Inc. (Hercules, CA). Fetal bovine serum (FBS) was purchased from Unipath (Bedford, UK). Monoclonal anti-rat clusterin antibody (mouse monoclonal IgG) specific for beta-chain was from UPSTATE Biotechnology (Lake Placid, NY). Apop Tag kit for in situ end labelling (ISEL) was from Oncor (MD, USA). CHO 1827 and CHO 1829 were provided by Serono International (Geneva, Swizzerland). Instagel plus was purchased from Packard (St Louis, MO). Finasteride (pure substance) (17β-(N,t-butyl)carbamoyl-4-aza-5α-androst-1-en-3-one) was a kind gift from Merck Sharp & Dohme Reaserch Laboratories (Rahway, NJ). Bicalutamide was a kind gift from AstraZeneca (AstraZeneca, Milan, Italy). Analogue 1-α-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol (Compound A)

was provided by Bioxell (Bioxell, Milan, Italy). Keratinocyte growth factor (KGF) was from Pepro Tech EC (London, England) and insulin-like growth factor-I, human, [Des(1-3)IGF-I] was purchased by GroPep Limited (Adelaide, Australia). In Situ Cell Death Detection Kit POD for terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick end-labelling (TUNEL) were from Roche Diagnostics Corporation (Indianapolis, IN). Plastic ware for cell cultures was purchased from Falcon (Oxnard, CA). Disposable filtration units for growth media preparation were purchased from PBI International (Milan, Italy). Lipofectamine 2000 and Opti-MEM I Medium for luciferase transfection were from Invitrogen, Life Technologies (San Giuliano Milanese, Milan, Italy). Thin-layer chromatography (TLC) silica plates were obtained from Merck (Darmstad, Germany). Testosterone enanthate (T enanthate) was from Geymonat (Anagni, Italy). Coat-A-Count® Total Testosterone detection kit was purchased from Medical System (Genova Struppa, Italy). Rat luteinizing hormone (rLH) [^{125}I] assay systems were from Amersham Pharmacia Biotech (Piscataway, NJ).

BPH cells

Human BPH cells, prepared, maintained and used as previously described in Crescioli C, *et al. Journal of Clinical and Endocrinology Metabolism* (2000) 85 p 2576-2583, were obtained from prostate tissues derived from 5 patients, who underwent suprapubic adenomectomy for BPH, after informed consent and approval by the Local Ethical Committee. Patients did not receive any pharmacological treatment in the 3 months preceding surgery.

5α reductase-transfected CHO-1827 and CHO-1829 cell lines

CHO-1827 and CHO-1829 cells, transfected with 5α reductase type 1 [illegible] described [illegible] Molec Cell Biol (2001) 55 p 17-

AR-transfected PC3 cell line

Human prostate adenocarcinoma PC3 cells, stably transfected with the plasmid p5HbhAR-A containing human androgen receptor (hAR) as previously described (see Bonaccorsi L, *et al. Endocrinology* (2000) 141 p 3172-3182), were grown in 75 cm^2 culture flasks in Ham's F-12 medium containing 50 µg/ml geneticine, 10% FCS, penicillin (100 U/ml) and streptomycin (100 mg/ml).

BPH tissue

Prostatic tissues for binding assay were obtained from patients who underwent suprapubic adenomectomy for BPH. No pharmacological treatment was performed in the 3 months preceding surgery. After surgery, the tissues were immediately placed in liquid nitrogen and stored at -80°C until processing.

Rat tissues

Rat ventral prostate glands were rapidly excised out, weighed and quickly frozen in dry ice. Immunohistochemistry experiments were performed in 14 µm-thick contiguous cryostatic sections for direct comparison of tissue morphology, clusterin expression and apoptosis localization by TUNEL. For total RNA extraction and Western blot analysis, rat ventral prostates from 4 to 6 animals were pooled.

BPH cell proliferation assay

For all cell proliferation assay, $4x10^4$ BPH cells were seeded onto 12-well plates in their growth medium, starved in red- and serum-free medium containing 0.1% BSA for 24 h, and then treated with specific stimuli for 48 h. Cells in phenol red- and serum-free medium containing 0.1% BSA were used as controls. Thereafter, cells were trypsinized, and each experimental point was derived from hemocytometer counting, averaging at least six different

fields for each well, as previously reported (see Crescioli C, *et al. Journal of Clinical and Endocrinology Metabolism* (2000) 85 p 2576-2583). Experiments were performed using increasing concentrations (10^{-18}-10^{-7}M) of calcitriol or Compound A with or without a fixed concentration of T (10 nM), KGF or Des(1-3)IGF-I (10 ng/ml). Growth assays were also carried out using a fixed concentration of androgens (10 nM) with or without Compound A (1 nM, 10 nM) or the anti-androgens finasteride (F, 1 nM) and cyproterone acetate (Cyp, 100 nM). Growth assays were also performed using a fixed concentration of T (10 nM) or GFs (10 ng/ml) with or without Compound A (10 nM). In the same experiment, each experimental point was repeated in triplicate or quadruplicate and experiments were performed 3 times. Results are expressed as % variation (mean±SEM) over the maximal T or GF-induced stimulation.

In situ end labeling (ISEL)

ISEL was performed onto BPH cells using Apop Tag in situ apoptosis detection kit peroxydase following the manufacturer's instruction. Cells were incubated with T (10 nM), KGF (10 ng/ml) or Des(1-3)IGF-I (10 ng/ml) with or without Compound A (10 nM). The percentage of apoptotic cells (the number of stained cells divided by the total number of cells) was calculated in at least five separate fields per slide in five different slides. Results are expressed as mean±SEM from three separate experiments.

5α reductase inhibition test

5α reductase inhibition assay was performed using CHO 1827 cells, transfected with 5αR-1, or CHO 1829 cells, transfected with 5αR-2, as described (see Guarna A, *et al. Journal of Medicinal Chemistry* (2000) 43 3713-3735.). Compound A was added in a concentration range from 10^{-9} to 10^{-} [illegible] 5α reductase activity in each experiment.

Binding assay on cytosol fractions of BPH fragments were carried out as previously reported (see Crescioli C *et al. Endocrinology* (2003) 144 p 3046-3057), (final protein concentration: 1.8 mg/ml). Incubations of cytosolic fractions were carried out with increasing concentration (0.125, 0.25, 0.5, 1 nM) of [^{3}H]-R1881 (specific activity: 83.5 Ci/mmol) in the absence or in the presence ([^{3}H]-R1881:1 nM) of increasing concentrations of cold R1881 (10^{-10}-10^{-6} M), DHT (10^{-10}-10^{-6} M), T (10^{-10}-10^{-6} M), bicalutamide (10^{-10}-10^{-4} M), and Compound A (10^{-10}-10^{-4} M). To prevent R1881 binding to progesterone receptor, 1 μM triamcinolone acetonide was added to each tube. Separation of bound and unbound ligand was performed as previously described (see Crescioli C *et al. Endocrinology* (2003) 144 p 3046-3057). Protein content was determined by the known method of Bradford, using BSA as a standard.

Luciferase assay

PC3 cells stably transfected with human AR were plated in 24-well plates at a density of 2x10^4 in Ham's F12 plus 10% FCS. After 24 hours, the cells were transfected with 750 ng/well of pLSPP plasmid containing the wilde-type sequence configuration of the MMTV-LTR linked to the firefly luciferase gene (see Pazzagli M. *et al. Analytical Biochemistry* (1992) 204 p 315-323.), using Lipofectamine 2000 (1 mg/ml) according to the manufacturer's instructions. After 48 h, the cells were incubated with DHT (10^{-12}-10^{-6} M) or bicalutamide (10^{-9}-10^{-5} M), in the presence of 3 nM of DHT, and with equimolar concentration of Compound A for 18 h. Steroids and Compound A analogue were dissolved in ethanol. Transfected cells incubated with ethanol only served as positive controls.

The Luciferase assay was performed with a Berthold luminometer according to the manufacturer's instructions (Luciferase Assay System, Promega, Milan, Italy). The cells were lysed directly in the plate with 200 μl of lysis buffer. Luciferase activity was measured on 20 μl of cell lysate for 10 s

after addition of 100 µl of luciferine. Total protein measurement was performed on 20 µl of cell lysate. At least three indipendent assays were done in duplicate.

Results

Incubation of BPH cells with increasing concentrations of calcitriol or Compound A inhibits cell growth (Figure 1 panel A). Both compounds inhibited dose-dependently cell proliferation. ALLFIT (see De Lean A, *et al. American Journal of Physiology* (1978) 235 p E97-E102) analysis indicated that, although maximal inhibition of calcitriol and Compound A ("Cmpd A" in the Figures) was not statistically significantly different (I_{max}=43±1%), their relative potency was, Compound A being several log units more effective than calcitriol (-logIC_{50} Compound A=15.8±0.3 vs -logIC_{50} calcitriol=10.2±0.6, P<0.005).

BPH cell proliferation was significantly increased (P<0.01) by testosterone (T) (156±8%), and growth factors (GF), such as Des(1-3)IGF-I (194±6%) or KGF (183±5%). When cell growth was stimulated for 48 h with T or GFs (Figure 1, panel B) the inhibitory effect of Compound A was even more pronounced (I_{max}=66.6±7.3%). Mathematical modelling (see De Lean A, *et al. American Journal of Physiology* (1978) 235 p E97-E102) of inhibition curves indicated that Compound A was more potent in BPH cells stimulated with T (-log IC_{50}s=16.4±0.6) than with the other two growth factors (-log IC_{50}s=12.7±0.6, and –log IC_{50}=14.2±0.6 for Des(1-3)IGF-I and KGF, respectively; P<0.0001).

Compound A (1 nM) antagonized not only T- but also DHT-stimulated BPH cell proliferation to an extent similar to the AR antagonist cyproterone acetate (CYP, 100 nM; Figure 2 panel A and B). Conversely, the 5α reductase

panel A). In addition, Compound A reduced growth even in androgen-unstimulated cells (Figure 2, panel A).

To evaluate potential anti-androgenic properties of Compound A, in addition to BPH cell growth inhibition, we investigated its interaction with the AR. First, we ruled out the possibility that Compound A binds to the AR by performing competition studies in human BPH homogenates, using the synthetic androgen [^{3}H]-R1881 as labelled ligand. LIGAND analysis (see Munson PJ et al. Analytical Biochemistry (1980) 107 p 220-239.) of the data indicated that unlabeled R1881, DHT, T, and the AR antagonist bicalutamide completely displaced [^{3}H]-R1881 binding (Table I). Conversely, Compound A did not compete for [^{3}H]-R1881 binding at any concentration tested (Table I). These results were confirmed and extended using a luciferase reporter gene assay. In PC3 cells expressing the full length AR coupled to a luciferase report gene, DHT stimulated a dose-dependent increase in luciferase activity (EC_{50}=2±1.3 nM, panel A), while bicalutamide inhibited DHT-stimulated activity (IC_{50}=194±80 nM, panel B). In this system, increasing concentration of Compound A neither stimulated nor inhibited AR-mediated luciferase activity increase (Figure 3). Finally to verify whether or not Compound A interacts with the formation of DHT, the active metabolite of T, we performed experiments in CHO cells transfected with type 1 and type 2 5α reductase. Results were compared to those obtained with finasteride (F). While F inhibited T conversion into DHT with the expected IC_{50}s, (IC_{50} for 5α reductase type 1 =659±100 nM and IC_{50} for 5α reductase type 2, =53.7±11 nM, n=3), Compound A did not interfere with either isoenzyme up to the micromolar range (data not shown).

AR Ligand	Affinity constants (K_d nmol/L)
R1881	0.16 ± 0.06
DHT	0.07 ± 0.03
T	1.89 ± 0.94
Bicalutamide	159 ± 82
Compound A	> 100000

Table I *Affinity constants of androgen agonists (R1881, DHT, T), antagonist (bicalutamide) and Compound A in human BPH homogenates as detected by [^{3}H]R1881 binding.*

The effect of Compound A in BPH cells was, at least in part, due to activation of programmed cell death as detected by ISEL (n=3, Table II). The percentage of apoptotic nuclei dramatically increased (270%) after a 48 h exposure to 10 nM Compound A ($P<0.01$ vs control). Conversely, treatment with T (10 nM), or GFs (10 ng/ml) significantly ($P<0.01$) reduced the number of apoptotic BPH cells as compared to untreated cells (Des(1-3)IGF-I= -42%; KGF=-54%; T= -27%). However, even in the presence of GFs or T, Compound A induced a sustained (more than 250%) and significant ($P<0.01$) increase in the number of ISEL-positive BPH cells.

Apototic index (%)

	control	Compound A
control	18.55±0.8	68.44±1.26 [a]
Des(1-3)IGF-I	10.69±0.6 [a]	45.85±0.66 [a, b, c]
KGF	8.5±0.42 [a]	44.46±0.57 [a, b, c]
T	13.56±0.72 [a]	49.06±1.87 [a, b, c]

Table II *Effect of Compound A (10 nM), GFs (10 ng/ml) or T (10 nM) on DNA fragmentation in BPH cells. Apoptotic index (%) represents the number of stained nuclei, as detected by ISEL, over BPH cells in each of at least 5 separate fields per slide. Results are expressed as mean±SEM in three separate experiments. Compound A is able to induce apoptosis in untreated BPH cells as well as in BPH cells simultaneously incubated with GFs or T (a: P<0.01 vs control; b: P<0.01 vs Compound A-treated cells; c: P<0.01 vs GF- or T-treated cells).*

Example 2: Anti-proliferative properties of Compound A in in vivo *models of prostate growth*

Animal protocols

Male Sprague Dawley rats (28 days old) were purchased from Charles River Laboratories (Calco, Lecco, Italy). All animal experimentation described was conducted in accord with accepted standards of animal care. Castration was performed via the scrotal route under ketamine/xylazine anaesthesia. Three days after castration, rats (5-8 animals per group) were treated or not with T enanthate (30 mg/Kg) in two separate weekly sc injections. Rats were orally treated for 5 days the first week, and 4 days the second week with vehicle (miglyol 812), Compound A (10, 30, 100 and 300 μg/Kg) or finasteride (10 and 40 mg/Kg) for a total of 9 administrations, and sacrificed one day later.

Alternatively, intact, adult male Sprague Dawley rats (weight 250 g) were dosed orally with vehicle (miglyol 812), Compound A (10, 30, 100 and 300 µg/Kg) or finasteride (10 and 40 mg/Kg) 5 days/week for 5 consecutive weeks and for two additional days the 6th week, for a total of 27 administrations, unless otherwise specified. Blood for calcium and hormone measurements was obtained at the end of each experimental protocol.

Northern Hybridisation analysis

Total RNA was extracted using RNAFast from Molecular Sysyem (San Diego, CA). Blotting, labelling, hybridization conditions and probes (rat clusterin 1.5 Kb full-length cDNA and GAPDH 1.2 Kb full-length cDNA) were performed according to the reported procedures (18, 19). Quantitation of the autoradiograms was obtained by densitometric scanning using an LKB Ultrascan XL densitometer.

Immunohistochemistry

All the cryostatic sections obtained from controls and treated rats were processed in parallel as previously described (20). For every experimental condition, 3 alternate sections from 3 different rat prostates were examined. Negative controls, made by excluding the specific antibody from the reaction, showed no specific staining. Counterstaining was performed with haematoxylin, and cover slips were mounted with Eukitt (O. Kindler GmbH & Co, Germany). Digital high-magnification colour images were acquired by a CCD camera through the microscope.

In situ DNA fragmentation analysis (TUNEL)

DNA fragmentation in prostate cryostatic sections, assessed by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end-labelling (TUNEL), was performed using the In Situ Cell Death Detection Kit, POD.

by a CCD camera through the microscope. Counterstaining was performed with eosin, and cover slips were mounted with Eukitt (O Kindler GmbH & Co, Germany).

Calcium measurements

Serum calcium levels were measured with a commercially available colorimetric assay (Sigma), according to the manufacturers' instructions.

Testosterone and rLH measurement

Serum levels of T and rLH hormones were determined by commercially available radioimmunoassay kits, according to the manufacturers' instructions. To measure serum T in rats, samples were first added to 4 volumes of diethyl ether, mixed by gentle inversion for 15 min and then centrifuged for 5 min at 2000 rpm. The aqueous phase was frozen in dry ice and the organic phase was recovered and evaporated to dryness under a nitrogen stream. The dried extract was reconstituted in the assay buffer as follows: (1 vol: 1 vol) in intact rat, and (4 vol: 1 vol) in castrated rats.

Statistical analysis

Statistical analysis was performed by one-way ANOVA and paired or unpaired Student's t tests, when appropriate. Binding data were analysed using the computerized program LIGAND (21).

The computer program ALLFIT (22) was used for the analysis of sigmoid dose-response curves to obtain estimates of half-maximal inhibition values (IC_{50}) and half-maximal stimulatory values (EC_{50}) as well as maximal inhibitory (I_{max}) and stimulatory (E_{max}) effects. Data were expressed as (mean±SEM).

Results

To test the anti-proliferative properties of Compound A in *in vivo* models of prostate growth, castrated and intact rats were orally treated with increasing

concentrations of Compound A (10-300 µg/Kg) or finasteride (F) (10, 40 mg/Kg). As shown in Figure 4, panel A, castration dramatically reduced ventral prostate weight, while a two-week treatment with testosterone (T) enanthate (30 mg/Kg) not only completely restored, but further stimulated its growth. Compound A, at any dose tested, completely blunted T-stimulated prostate over-growth, reducing ventral prostate weight below that of untreated rats. Similar results were obtained with finasteride (10, 40 mg/Kg). A one-month treatment of intact adult rats with Compound A significantly decreased ventral prostate weight, with a maximal reduction (30%) at the highest dose tested (300 µg/Kg). At this dose, the inhibitory effect of Compound A on prostate growth was comparable to that induced by 10 or 40 mg/Kg finasteride (Figure 4, panel B). In all the experimental protocols, oral administration of different doses of Compound A caused a very modest hypercalcemia only at the highest dose tested (300 µg/Kg) (Table III). No other discernible side effects were observed.

	calcemia
Control	10.2 ± 0.16
Compound A 10 µg/Kg	10.16 ± 0.24
Compound A 30 µg/Kg	9.87 ± 0.15
Compound A 100 µg/Kg	10.55 ± 0.18
Compound A 300 µg/Kg	10.85 ± 0.1

Table III *Calcemia (mg/dl) in T-replaced castrated rats after different doses (10, 30, 100, 300 µg/Kg) of Compound A. Compound A never changed calcium serum levels in castrated rats replaced with T enanthate (30 mg/Kg/week) as compared to controls. Similar results were obtained in intact rats (not shown). Results represent the mean±SEM of rats/group.*

To better understand the molecular mechanisms underlying Compound A-induced prostate weight reduction, the expression of clusterin gene and

(TUNEL). Clusterin is an ubiquitous product gene, strictly related to cell cycle arrest and atrophy, the expression of which is down regulated by androgens. Figure 5 panel A, shows the prostatic expression of clusterin mRNA, as detected by Northern analysis, in orchidectomized rats supplemented or not supplemented with T. Castration dramatically up-regulated clusterin mRNA abundance, while this effect was completely reverted by a two-week administration of T. The simultaneous treatment with different concentrations of Compound A (300 and 100 μg/Kg) or F (40 mg/Kg) partially blunted the T-induced down-regulation of clusterin gene expression. In intact rats (Figure 5 panel B), a one-month administration of different concentrations of Compound A (30 and 100 μg/Kg) induced a sustained increase in clusterin gene expression in the prostate, comparable, or even higher, than that induced by 40 mg/Kg F.

The local expression of clusterin in the prostate of orchidectomized rats is shown in Figure 6. Castration induced a marked and widespread atrophy in the prostate gland, and nearly all the cuboidal epithelial cells facing the gland lumen were clusterin positive (panel A). T-replacement (panel B) reverted the morphological hallmarks of atrophy and consistently reduced clusterin staining. Such an effect was prevented by the simultaneous administration of Compound A (panels C and E). Panels D and F show TUNEL results in sections adjacent to those shown in panels C and E. Compound A treatment (100 μg/Kg, panel D and 300 μg/Kg, panel F) induced an evident nuclear fragmentation in epithelial and stromal cells, and apoptosis was detectable in both clusterin positive and negative cells. The first two panels of Figure 7 show the morphology of the prostate gland of an intact rats processed for clusterin detection, with (panel A) or without (panel B) the omission of the primary antibody. Note that clusterin labelling is almost absent in the prostate of untreated adult rats (panel B), as it is nuclear fragmentation (TUNEL, panel C). Conversely, treatment with different doses of Compound A induced clusterin

expression (panels D-F) and apoptosis (C, G and I). Panel F shows, for comparison, the effect of finasteride (40 mg/Kg) on clusterin positivity in the prostate gland.

To rule out the possibility that Compound A reduced *in vivo* prostate growth by interfering with pituitary or testis function, rat luteinizing hormone (rLH) and T serum levels were measured in castrated and intact rats. As expected (Table IV, panel A), castration significantly reduced T while it increased rLH serum levels. T enanthate (30 mg/Kg) administration (two weeks) completely reverted the effect of orchidectomy. Oral treatment with Compound A (100 and 300 μg/Kg) of T-replaced castrated rats did not significantly affect rLH or T serum levels. Similar results were obtained in intact rats (Table IV, panel B). In fact, chronic administration (1 month) of Compound A (10, 30, 100 μg/Kg) or F (40 mg/Kg) to intact rats did not modify rLH and T serum levels.

Panel A

	rLH	T
control (intact rats)	2.36 ± 0.46	11.5 ± 2.44
Castrated	20.64 ± 6*	0.9 ± 0.32*
castrated+T-replaced	2.08 ± 0.36	21.25 ± 4.12
castrated+T-replaced +Compound A 100 µg/Kg	1.8 ± 0.2	11.13 ± 1.02
castrated+T-replaced +Compound A 300 µg/Kg	3.15 ± 0.65	15.73 ± 2.75

Panel B

	rLH	T
control (intact rats)	2 ± 0.16	11.98 ± 2.87
Finasteride	2.2 ± 0.4	18.11 ± 3.23
Compound A 10 µg/Kg	2.22 ± 0.25	19.13 ± 3.83
Compound A 30 µg/Kg	2.32 ± 0.36	9.39 ± 2
Compound A 100 µg/Kg	1.96 ± 0.13	11 ± 2.14

Table IV *rLH (ng/ml) and T (nM) serum levels in T-replaced castrated (panel A) or intact (panel B) rats after treatment with different doses of Compound A. Panel A. Castration significantly reduced serum T (*P<0.01 vs control) while it increased serum rLH (*P<0.05 vs control). After treatment with T enanthate (30 mg/Kg/week) rLH and T serum levels were restored. Compound A at all the doses tested did not significantly affect either rLH or T serum levels. Panel B chronic administration (1 month) of F (40 mg/Kg) or Compound A (10, 30 and 100 µg/Kg) did change neither rLH nor T serum levels in intact rats.*

This study demonstrates that Compound A reduces prostate size in intact rats to an extent similar to finasteride. In addition, as finasteride, Compound A abolishes the *in vitro* and *in vivo* proliferative activity of testosterone. However, at variance with finasteride, Compound A does not inhibit type-1 or type-2 5α-reductase activity and can counteract not only T but even DHT induced BPH cell growth. These anti-androgenic properties of Compound A are independent from interaction with the AR, as shown by the failure of Compound A to bind to

the AR, and to act as AR agonist or antagonist in AR-transfected PC3 cells. Furthermore, Compound A does not affect sex hormone secretion because, in the rat, gonadotropin and T plasma levels were unchanged by daily administration of Compound A for up to one month. Hence, Compound A acts downstream the AR receptor-ligand interaction. Without wishing to be bound by theory this action most probably occurs via the disruption of testosterone-growth factor cross talk.

Very low concentrations of Compound A were able to completely antagonize not only T-stimulated BPH cell proliferation, but also proliferation induced by the two most important intra-prostatic growth factors: IGF-I and KGF. In addition, even in the presence of T or GFs, Compound A induced apoptosis in BPH cells. The Compound A-induced death program was evident also in the prostate of both intact and T-supplemented orchidectomized rats and was characterized by the diffuse appearance of DNA fragmentation with a concomitant increase in clusterin gene and protein expression. Clusterin is a protein tightly regulated in the prostate by androgens (18). Although clusterin function is still not well understood, it is markedly up-regulated in conditions of gland atrophy (23, 25) and apoptosis (33). Thus, clusterin induction by Compound A treatment is consistent with the capacity of this compound to inhibit proliferation and induce apoptosis in prostate cells.

In conclusion, this study indicates that Compound A is effective in reducing prostate cell growth in different experimental models.

Example 3: Reduction of prostate weight in healthy dogs treated with Compound A.

A 9-month toxicity study was carried out in four groups of male beagle dogs, which were treated by daily oral gavage with 0.5 µg, 1.5 µg and 5 µg/kg body weight/day of Compound A (in vehicle Miglyol 812) or with vehicle alone. This treatment was followed by a 2-month recovery period for the group receiving the highest dose, 5 µg, after which prostate weights was measured. In addition to entirely favourable toxicity data, a lower prostate weight was observed at the end of treatment with Compound A (see Figure 8) and after recovery (see Figure 9). The results after recovery were analysed statistically via a one-tailed Student's t test and were found to be significantly different between Compound A and vehicle ($P<0.05$). These results further demonstrate the ability of Compound A to reduce prostate size *in vivo*.

Example 4: Oral Dosage Form Soft Gelatin Capsule

A capsule for oral administration is formulated under nitrogen in amber light from 0.01 to 25.0 mg of one of the compounds of the present invention in 150 mg of fractionated coconut oil, with 0.015 mg butylated hydroxytoluene (BHT) and 0.015 mg butylated hydroxyanisole (BHA), filled in a soft gelatin capsule.

CLAIMS

1. The use of 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol for the manufacture of a medicament for the prevention and/or treatment of benign prostatic hyperplasia.

2. The use of 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol in combination with a second BPH-active agent for the manufacture of a medicament for the prevention and/or treatment of benign prostatic hyperplasia.

3. The use of claim 2 wherein said second BPH-active agent is an alpha-adrenergic receptor blocking agent.

4. A pharmaceutical composition comprising 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol and a pharmaceutically acceptable excipient for the treatment and/or prevention of benign prostatic hyperplasia.

5. The invention as hereinbefore described.

FIGURES

Figure 1A.

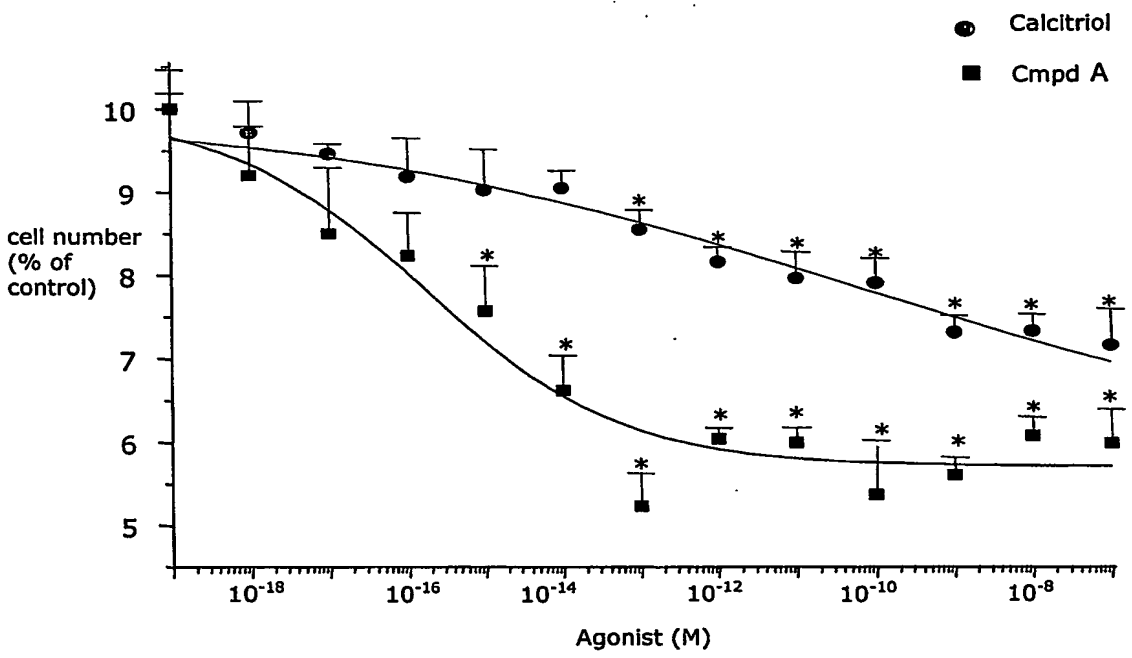


Figure 1B.

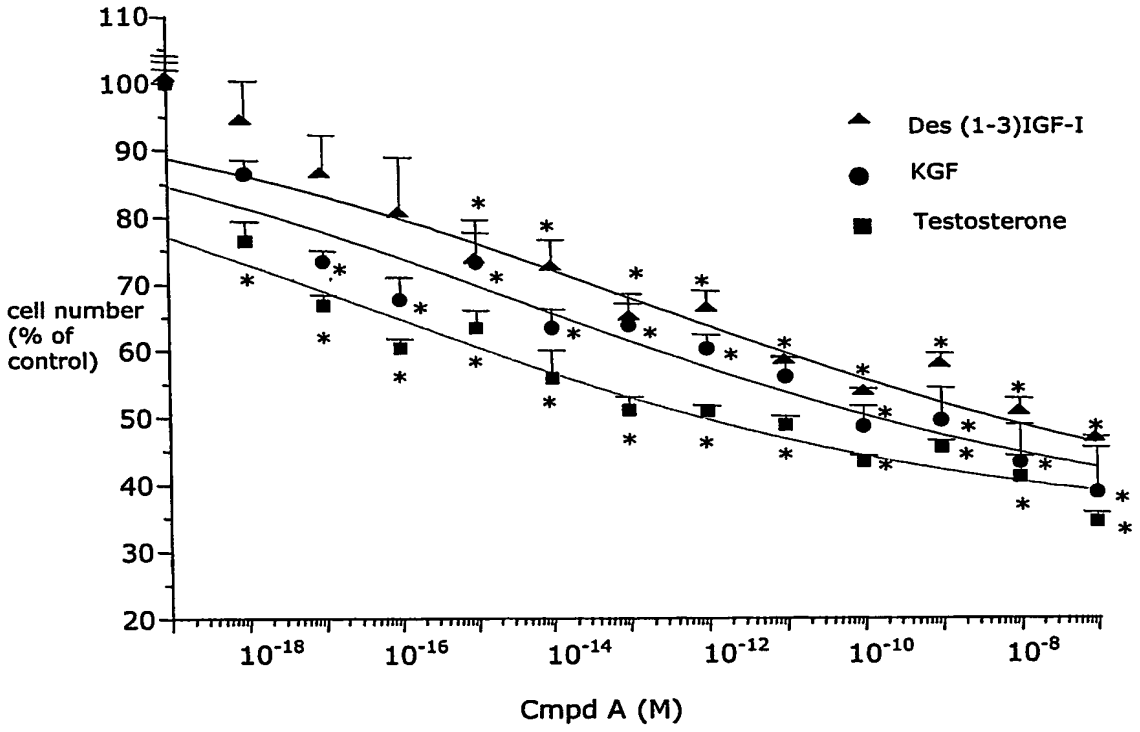

110
100
90
80
70
60
50
40
30
20
cell number (% of control)
Des (1-3)IGF-I
KGF
Testosterone
10^{-18}
10^{-16}
10^{-14}
10^{-12}
10^{-10}
10^{-8}
Cmpd A (M)

Figure 2A.

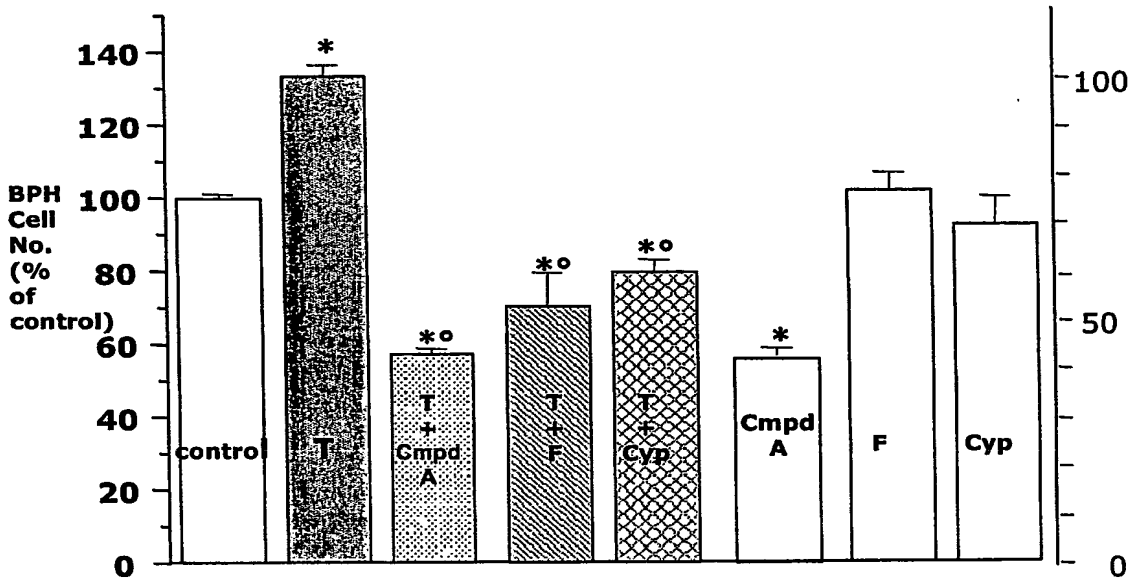

BPH Cell No. (% of control)
140
120
100
80
60
40
20
0
100
50
0
control
T
T + Cmpd A
T + F
T + Cyp
Cmpd A
F
Cyp
*
*°

Figure 2B.

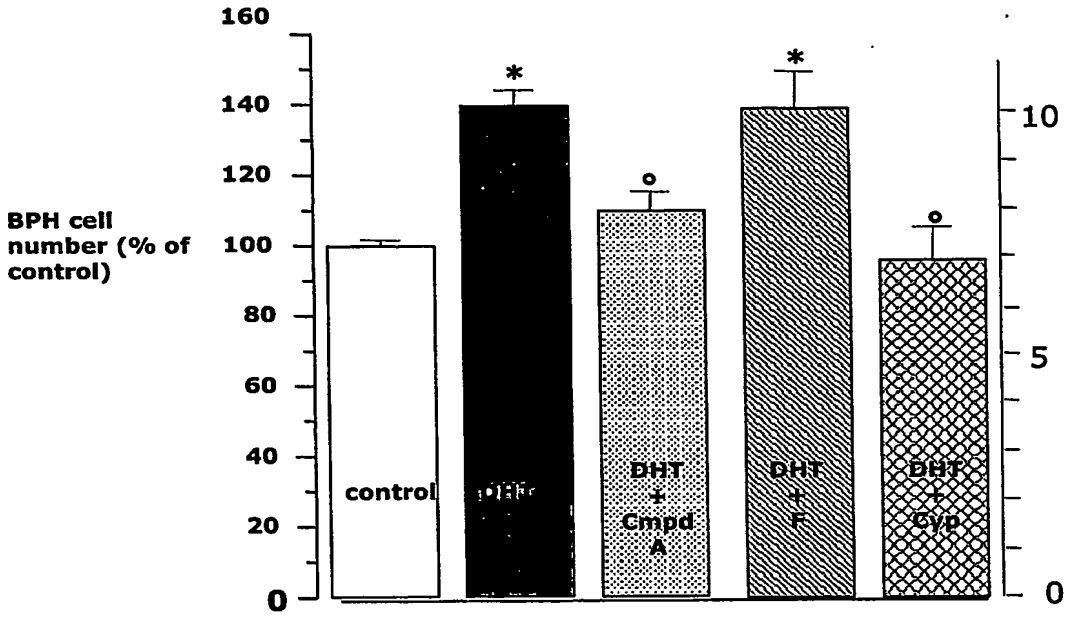

BPH cell number (% of control)
160
140
120
100
80
60
40
20
0
10
5
0
*
*
°
°
control
DHT
DHT + Cmpd A
DHT + F
DHT + Cyp

Figure 3A.

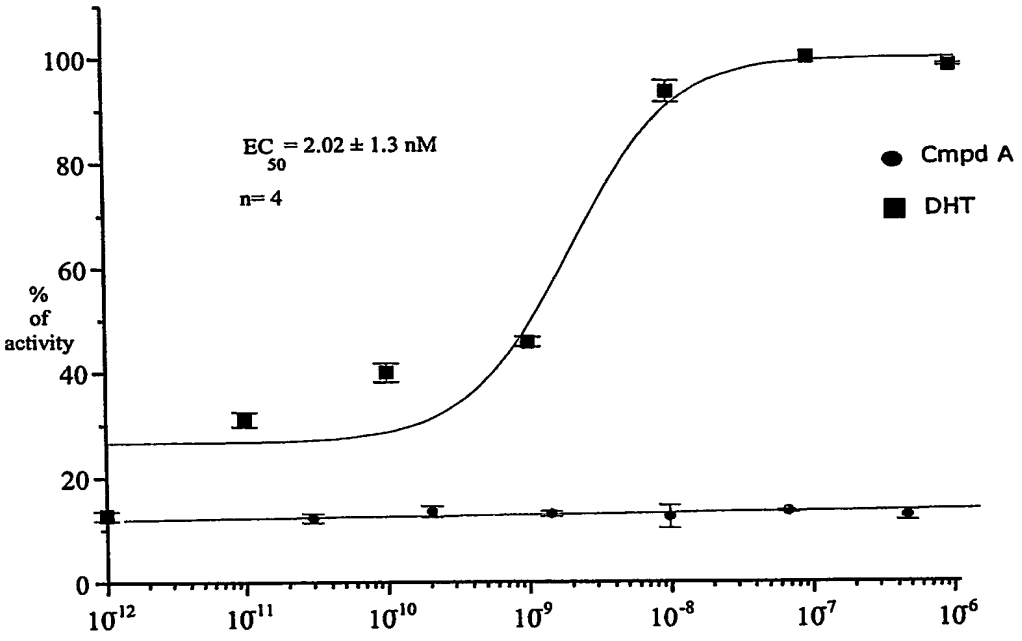

100
80
60
40
20
0
% of activity
EC 50 = 2.02 ± 1.3 nM
n= 4
Cmpd A
DHT
10-12
10-11
10-10
10-9
10-8
10-7
10-6

Figure 3B.

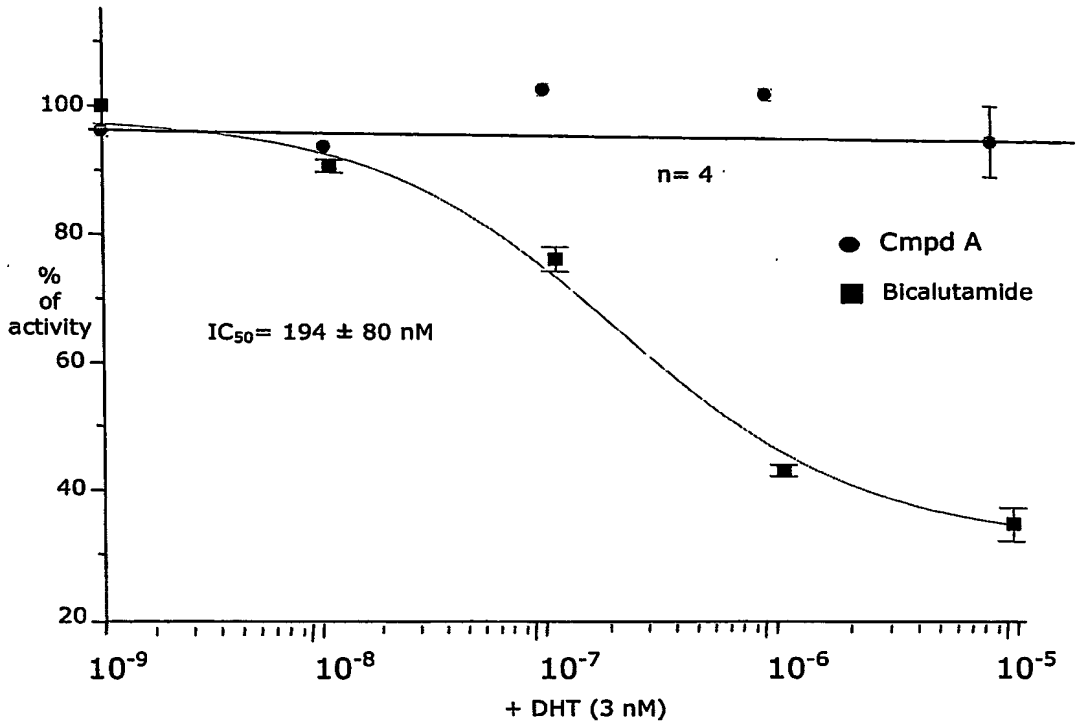

100
80
60
40
20
% of activity
n= 4
Cmpd A
Bicalutamide
IC50= 194 ± 80 nM
10-9
10-8
10-7
10-6
10-5
+ DHT (3 nM)

Figure 4A.

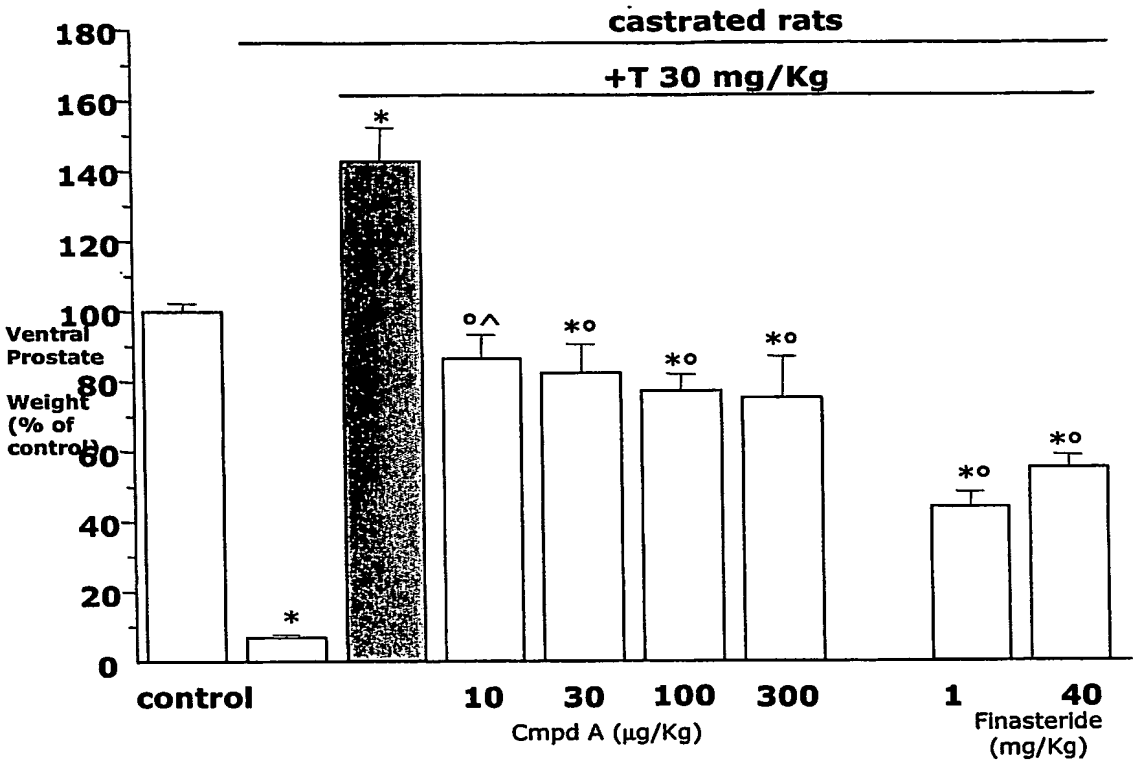

castrated rats
+T 30 mg/Kg
Ventral Prostate Weight (% of control)
180
160
140
120
100
80
60
40
20
0
control
10
30
100
300
1
40
Cmpd A (µg/Kg)
Finasteride (mg/Kg)

Figure 4B.

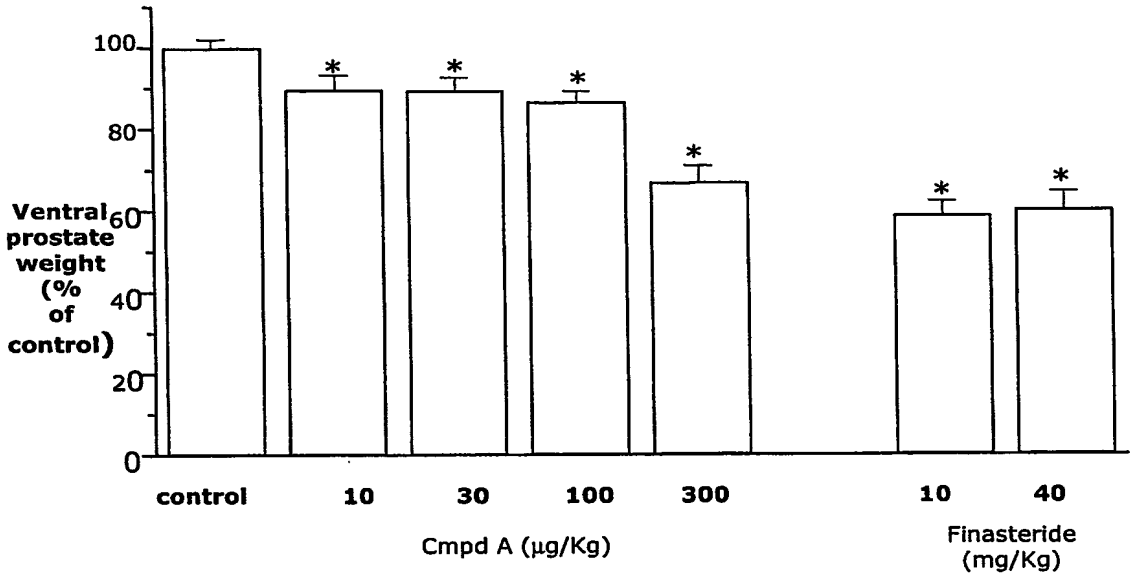

100
80
60
40
20
0
Ventral prostate weight (% of control)
control
10
30
100
300
10
40
Cmpd A (µg/Kg)
Finasteride (mg/Kg)

Figure 5.

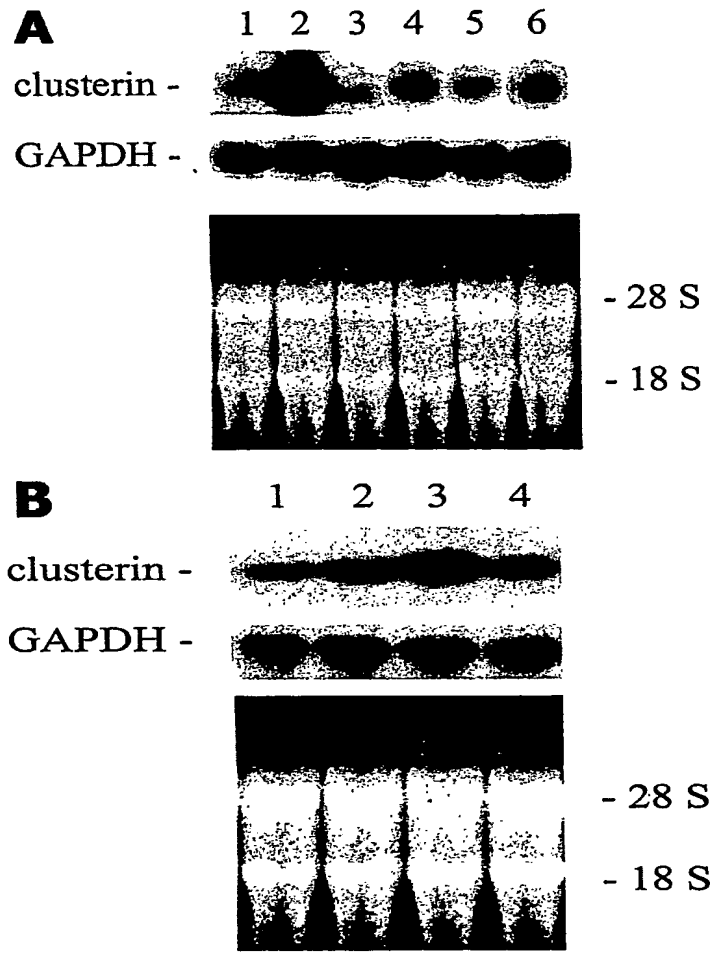

A
1 2 3 4 5 6
clusterin -
GAPDH -
- 28 S
- 18 S
B
1 2 3 4
clusterin -
GAPDH -
- 28 S
- 18 S

Figure 6.

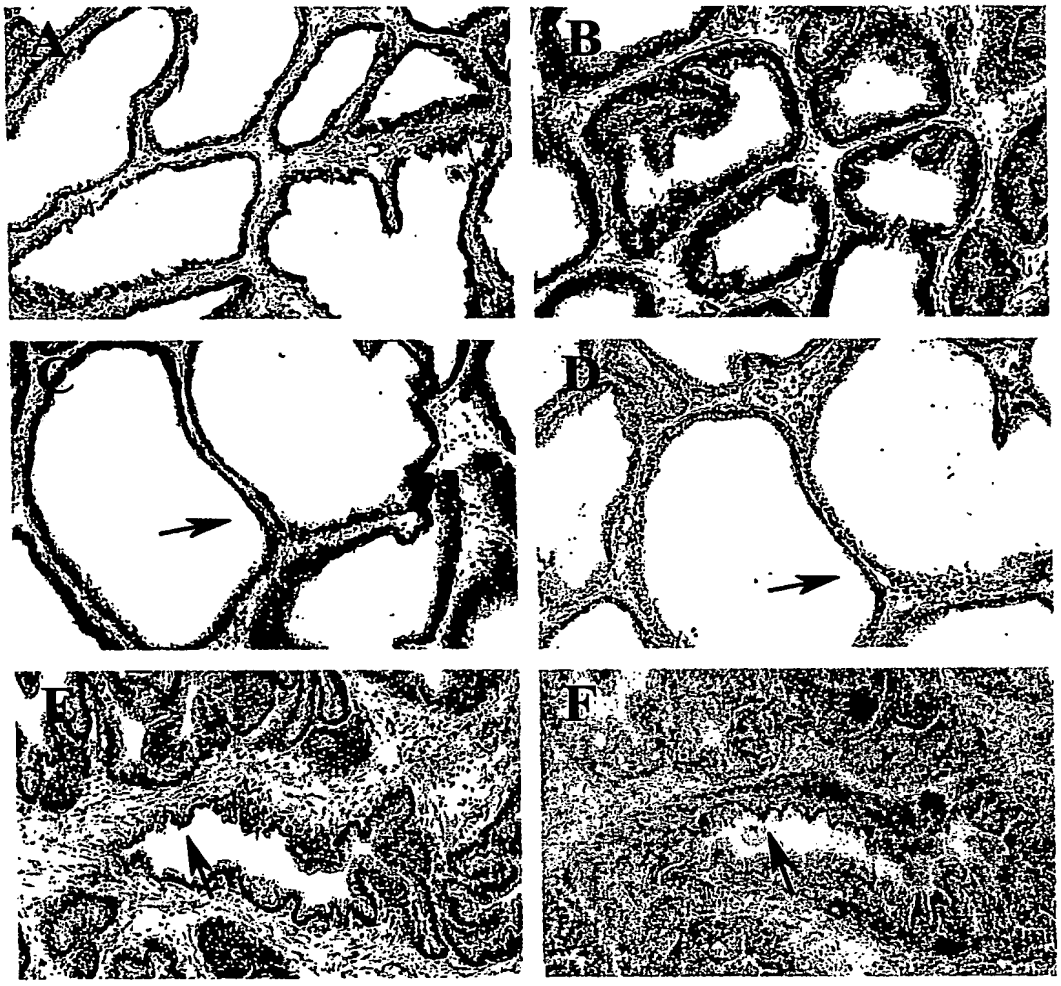


Figure 7.

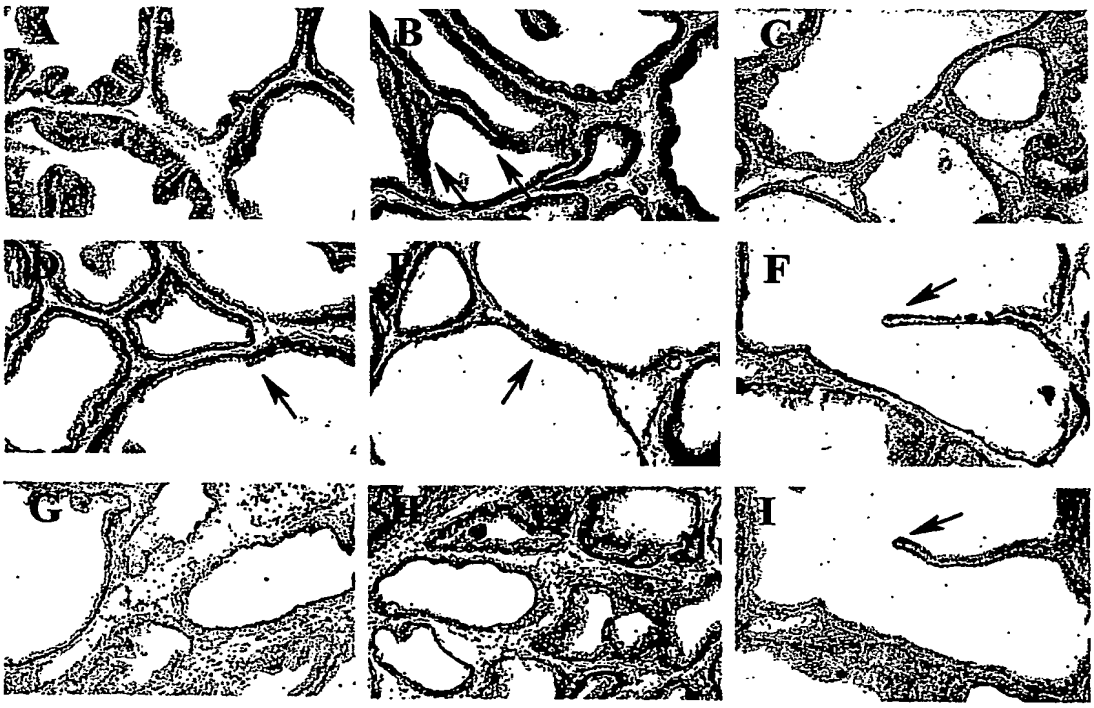
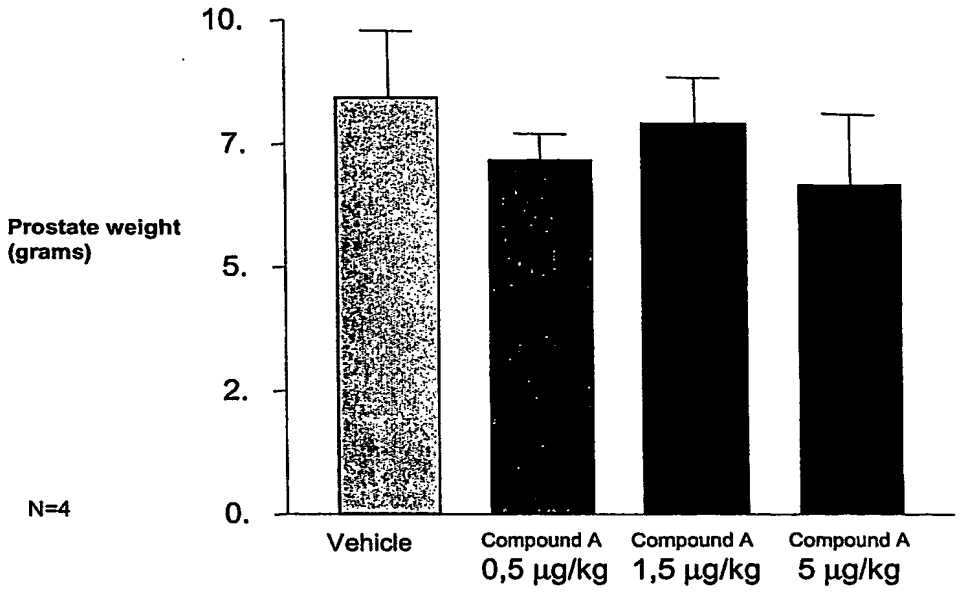
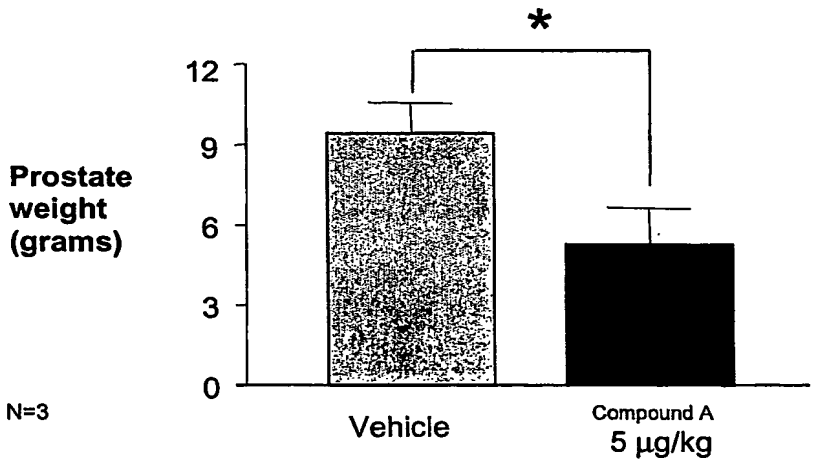


Figure 8.



Percent reduction	15	6.4	21

Figure 9.



Percent reduction 44